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**Authors**

Wang, KW

Tai, KK

Goldstein, SA

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# Mink Residues Line a Potassium Channel Pore

Ke-Wei Wang,<sup>†‡</sup> Kwok-Keung Tai,<sup>†‡</sup>  
and Steve A.N. Goldstein\*

\*Department of Pediatrics

<sup>†</sup>Department of Cellular and Molecular Physiology  
Boyer Center for Molecular Medicine  
Yale University School of Medicine  
New Haven, Connecticut 06536

## Summary

MinK has neither the P region nor signature sequence that characterizes pore-forming subunits of all known K<sup>+</sup> channels. A specific minK region has now been identified that affects external blockade by 2 common probes of K<sup>+</sup> channel pores. When mutated to cysteine, residues in this region render minK susceptible to covalent blockade by methanethiosulfonate ethylsulfonate and alter reversible inhibition by tetraethylammonium. The 2 blockers are found to share overlapping binding site determinants and to interact. Since inhibition by external tetraethylammonium is sensitive to voltage and to the internal concentration of permeant ions, we argue that tetraethylammonium blocks by occluding the external end of a water-filled transmembrane pore. These findings support the view that minK is directly involved in forming a K<sup>+</sup>-selective ion conduction pathway.

## Introduction

The pore of an ion channel determines its selectivity and conduction characteristics, and residues that line channel pores have been sought to study the molecular basis for channel function (Hille, 1992). One successful method has been to screen site-specific channel mutants for residues that alter blockade by agents that physically occlude the ion conduction pathway (Miller, 1995). Two agents used to study voltage-gated K<sup>+</sup> channels are methanethiosulfonate-ethylsulfonate (MTSES), which reacts covalently with exposed cysteines (Akabas et al., 1992; Kurz et al., 1995; Pascual et al., 1995), and tetraethylammonium (TEA), a reversible open-channel pore blocker (MacKinnon and Yellen, 1990; Yellen et al., 1991).

MinK is found in the plasma membrane of cardiac and auditory cells where it appears to mediate a slowly activating, voltage-dependent, K<sup>+</sup>-selective current (Folander et al., 1990; Honore et al., 1991; Sakagami et al., 1991; Freeman and Kass, 1993; Varnum et al., 1993; Marcus and Shen, 1994). Expression of the protein in *Xenopus laevis* oocytes induces a current with similar kinetics, selectivity, pharmacology, and regulation (Takumi et al., 1988; Hausdorff et al., 1991; Blumenthal and Kaczmarek, 1992; Swanson et al., 1993; Varnum et al., 1993). Yet, minK has just 130 amino acids and shows

no homology with known K<sup>+</sup> channels to suggest the molecular basis for its function.

MinK possesses a single central membrane-spanning segment (Figure 1) (Takumi et al., 1991; Busch et al., 1992a; Blumenthal and Kaczmarek, 1994). This segment is among 50 core residues that are implicated in the protein's function—first, by their striking conservation in the four species from which minK has been isolated (96% identity) and, second, by the effects of their mutation (Murai et al., 1989; Goldstein and Miller, 1991; Takumi et al., 1991; Swanson et al., 1993; Wang and Goldstein, 1995). Previously, we identified two residues in this segment that influence the permeability of NH<sub>4</sub><sup>+</sup> and Cs<sup>+</sup> relative to K<sup>+</sup> when mutated (Goldstein and Miller, 1991). Although this suggested the proximity of minK positions 55 and 59 to an ion channel selectivity determining region, it did not show that minK acted directly on permeant ions or that it lined an ion conduction pathway. Others have argued against any direct role for minK in K<sup>+</sup> transport (Attali et al., 1993).

In this paper, we study 14 consecutive minK positions thought to cross from the extracellular solution into the plasma membrane. Once mutated to cysteine and expressed in *Xenopus laevis* oocytes, the influence of each position on block of minK currents is assessed. We find the following: first, covalent modification of cysteine at 3 minK positions by MTSES blocks current; second, mutation at 5 positions alters block by TEA; third, TEA appears to block minK by a direct pore-occlusion mechanism; and, fourth, TEA can protect minK from covalent modification by MTSES. We conclude that minK directly lines the pore of a voltage-gated K<sup>+</sup> channel.

## Results

### MTSES Blocks a Subset of MinK Cysteine Mutants

As potential minK probes, water-soluble, membrane-impermeant, sulfhydryl-specific methanethiosulfonate derivatives were screened (Akabas et al., 1992; Stauffer and Karlin, 1994). Methanethiosulfonate-ethylammonium (MTSEA), a positively charged reagent used successfully with cysteine point mutants of other K<sup>+</sup> channels, was found to be an inadequate probe for minK. MTSEA irreversibly blocks both wild-type minK and cysteine-free C107A-minK in which the single native cysteine in minK is mutated to alanine (see Experimental Procedures); this appears to result from modification of the cysteine in a non-minK protein that is critical for minK channel function (Tai et al., 1996). On the other hand, the negatively charged reagent MTSES blocks neither wild-type minK nor C107A-minK and is thus a useful scanning agent (Table 1).

To identify residues that might contribute to a channel pore, minK positions 42–55 were mutated individually to cysteine in the otherwise cysteine-free C107A protein. Mutation of positions 44, 45, and 47 renders minK sensitive to irreversible blockade by MTSES (Figures 2A and 2B). Cysteine substitution of the other positions produces functional channels that, like L43C, L46C, and

<sup>‡</sup> These authors contributed equally to this work.

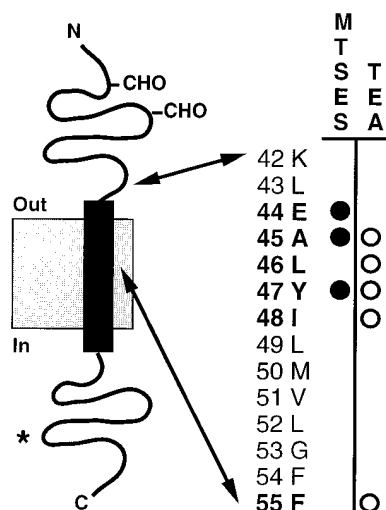


Figure 1. Proposed Membrane Topology for MinK

Indicated are the amino (N) and carboxyl (C) termini, 2 N-linked glycosylation sites (CHO), the highly conserved central 50 amino acids (boxed, positions 42–91 of rat minK) and the single natural cysteine (asterisk indicates position 107). Single-letter codes are given for the amino acids studied in this report. Positions in bold are those identified here to alter MTSES (closed circles) or TEA binding (open circles).

I48C-minK (Figure 2A) or C107A-minK (Table 1), exhibit wild-type  $K^+$  selectivity, gating, and insensitivity to MTSES.

The kinetics of MTSES blockade can be studied by abrupt exposure of oocytes expressing A45C-minK to

1, 3, and 10 mM MTSES (Figure 3). Though exposure to higher concentrations of MTSES results in more rapid development of block, the same final fraction of block is achieved at all 3 levels. In all experiments, block is irreversible and shows no change with washout of MTSES (Figure 3). Varying test-pulse duration from 2 s to 3 min (40 oocytes), interpulse duration from 0 to 3 min (46 oocytes), and command voltage from  $-10$  to  $40$  mV (44 oocytes) has no effect on the time course or magnitude of block of A45C-minK channels. This suggests that position 45 is exposed to the external aqueous milieu in both open and closed channels and occupies a site superficial to the transmembrane electric field. This is not unexpected: MTSES is negatively charged and unlikely to enter deeply into a cation-selective channel (Akabas et al., 1992).

MTSES inhibition of A45C-minK channels is not due to changes in channel gating kinetics (Table 1). This suggests that block is direct and not via allosteric effects on protein conformation. The notion that MTSES blocks minK via direct pore occlusion is compatible with its proposed mode of action on two Shaker-type  $K^+$  channels, the acetylcholine receptor, the cystic fibrosis transmembrane conductance regulator, and the  $\gamma$ -aminobutyric acid receptor (Akabas et al., 1992, 1994a, 1994b; Xu and Akabas, 1993; Kurz et al., 1995; Pascual et al., 1995). However, in the absence of voltage or state effects commonly associated with a pore blockade, we sought further support for this mechanism.

#### TEA Blocks MinK via Direct Pore Occlusion

TEA blocks a wide variety of potassium channels in the millimolar affinity range by direct pore occlusion

Table 1. Potassium Selectivity, Gating Kinetics, and Blocking Parameters of Wild-Type and Mutant MinKs

	Wild-Type	C107A	A45C	A45C + MTSES
$\Delta V_{rev}$ (2–20 mM KCl)	$59 \pm 3$	$63 \pm 2$	$61 \pm 2$	$62 \pm 1$
Activation				
$I_{10}/I_{25}$	$2.9 \pm 0.3$	$2.3 \pm 0.2$	$2.6 \pm 0.2$	$3.2 \pm 0.2$
Deactivation				
$\tau_1$	$1028 \pm 83$	$953 \pm 70$	$1031 \pm 44$	$1189 \pm 11$
$\tau_2$	$3150 \pm 63$	$2909 \pm 155$	$3164 \pm 46$	$3029 \pm 42$
$A_1/(A_1 + A_2)$	$0.13 \pm 0.02$	$0.14 \pm 0.02$	$0.08 \pm 0.04$	$0.10 \pm 0.04$
$Ba^{2+}$				
$K_i$ (mM, 20 mV)	$3.1 \pm 0.3$	$2.5 \pm 0.5$	$3.1 \pm 0.4$	$2.5 \pm 0.7$
TEA				
$K_i$ (mM, $-30$ mV)	$109 \pm 10$	$120 \pm 12$	$103 \pm 9$	$160 \pm 27$
$z\delta$	$0.18 \pm 0.06$	$0.16 \pm 0.03$	$0.17 \pm 0.04$	$0.20 \pm 0.02$
MTSES 10 mM				
$\tau_{1/2}$	ND	ND	$52 \pm 8$	ND
$f_{bl}$	$-0.02 \pm 0.02$	$-0.01 \pm 0.03$	$0.46 \pm 0.07$	

Tail current reversal potentials and voltage-dependent blockade were studied by opening channels with a 10 s pulse to 20 mV and measuring current 25 ms after shifting to test potentials of from  $-110$  to  $-10$  mV ( $V_{rev}$ ) or  $-50$  to  $10$  mV (block); electrical distance ( $\delta$ ) was calculated from  $K_i(V) = K_i(0)\exp(z\delta FV/RT)$ , where  $K_i(0)$  is the zero-voltage inhibition constant,  $z$  is the valence of the blocking ion and  $\delta$  is the fraction of the applied voltage drop experienced at the blocker's binding site, as described (Goldstein and Miller, 1991). Activation kinetics were evaluated by opening channels with a 10 s test pulse to 20 mV from  $-80$  mV and taking the ratio of macroscopic currents at 10 and 2 s; deactivation kinetics were studied by the same protocol at  $-70$  mV in the presence of 20 mM external KCl. Deactivation was fit according to  $I(t) = A_0 + A_1(\exp(-t/\tau_1) + A_2 \exp(-t/\tau_2))$  where  $A_0$  represents the current at steady-state, and  $A_1$  and  $A_2$  the amplitudes of the components represented by time constants  $\tau_1$  and  $\tau_2$ , respectively. Inhibition constants ( $K_i$ ) were determined after lead subtraction as described (Goldstein and Miller, 1991). The time constant for MTSES block ( $\tau$ ) and fraction of unblocked current ( $f_{bl}$ ) were estimated as in Figures 3 and 6 and  $\tau$  found to be  $197 \pm 33$  s (3 oocytes) and  $86 \pm 5$  s (9 oocytes) for 1 and 3 mM MTSES, respectively. Test solutions were ND-96 in which NaCl was isotonicity substituted by 18 mM KCl, 5 mM barium chloride ( $Ba^{2+}$ ), 75 mM tetraethylammonium chloride (TEA), or 10 mM sodium MTSES. Values are mean  $\pm$  SD for 4–7 oocytes.

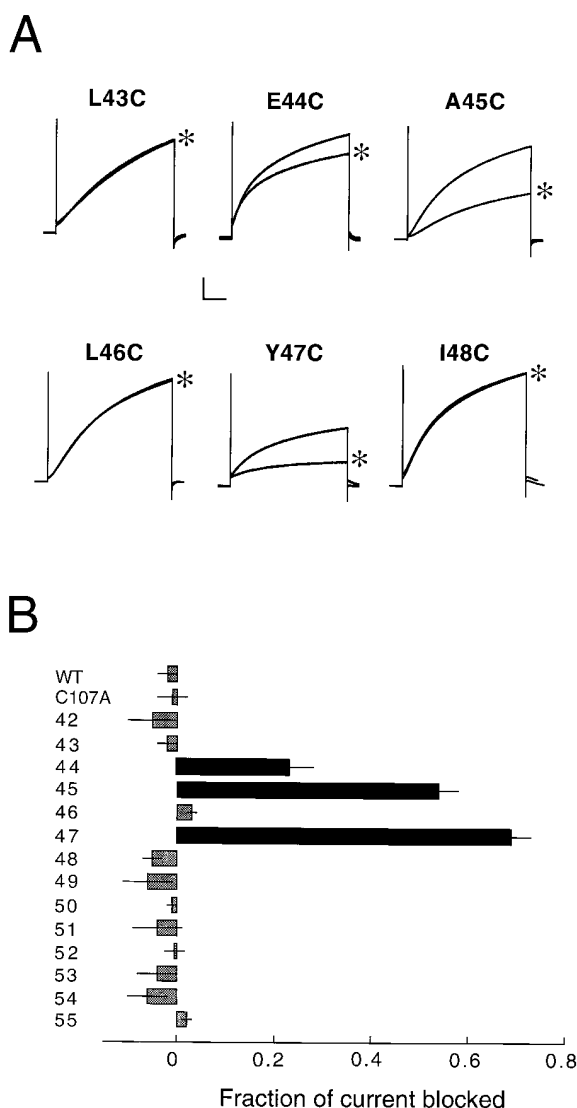


Figure 2. MTSES Block of MinK Cysteine Mutants

(A) Current traces from several minK mutants with cysteine in the indicated position before and after (asterisks) exposure to 10 mM MTSES for 3 min. Traces show 10 s test pulses to 20 mV from a holding potential of  $-80$  mV by two-electrode voltage clamp with constant flow of ND-96; repetitive test pulses were applied with a 15 s interpulse interval for the duration of the experiment. Scale bars represent  $0.1 \mu\text{A}$  (L43C, E44C, and Y47C) or  $0.2 \mu\text{A}$  and 2 s. (B) Susceptibility of cysteine-substituted minK mutants to MTSES. Current was measured at the end of each pulse by the protocol of (A). Bars indicate the fraction of blocked current ( $1 - I/I_0$ ), where  $I$  is the measured current after MTSES and  $I_0$  the current before MTSES, for 4–20 oocytes (mean  $\pm$  SD). Solid bars, mutants for which block was significantly different ( $p < .05$ ) than wild type (WT) and C107A-minK.

(MacKinnon and Yellen, 1990). Two lines of evidence indicate that TEA block of minK channels occurs by the same mechanism. First, TEA acts in a voltage-sensitive manner (Goldstein and Miller, 1991). TEA blocks A45C and wild-type minK as if partially penetrating the electric field to a receptor site  $\sim 15\%$  of the way across the transmembrane potential drop (Table 1). Second, TEA

inhibition of minK is sensitive to increased  $\text{K}^+$  concentration on the opposite side of the membrane. This is shown by microinjection of KCl into oocytes, an approach developed by others to study regulation of oocyte protein synthesis (Lau et al., 1988). Microinjection of 5–20 nmol KCl into oocytes expressing minK leads to a marked decrease in external TEA blockade (Figure 4). The new equilibrium dissociation constants ( $K_i$ ) are linearly dependent on KCl dose and stable from 30 to 120 min after microinjection. Microinjection of 20 nmol KCl shifts the minK reversal potential to more hyperpolarized potentials ( $-13 \pm 2$  mV, 3 oocytes) and increases the magnitude of outward minK currents ( $51\% \pm 13\%$ , 3 oocytes). Microinjection of rubidium chloride also diminishes external TEA affinity (Figure 5), shifts reversal potential, and increases outward currents (data not shown). Cesium chloride has only small effects, whereas sodium chloride is inactive (Figures 4 and 5). Transition effects in other potassium channels are thought to indicate entry of internal permeant ions into the conduction pathway and subsequent destabilization of the external pore blocker on its site (Armstrong, 1969; MacKinnon and Miller, 1988). Our findings are concordant with this idea since each injected cation decreases external TEA affinity according to its position in the relative permeability series determined for open minK channels (Goldstein and Miller, 1991). TEA thus appears to bind in the external minK channel pore, a region we postulated to mediate MTSES blockade. This suggests that sites for the 2 blockers might overlap.

#### TEA and MTSES Interact Negatively

When A45C-minK channels are blocked first by TEA and then abruptly exposed to MTSES (in the continued presence of TEA), the rate of irreversible MTSES blockade is markedly slowed (Figure 6A). As TEA concentration increases, the time constant of MTSES block increases linearly while the final magnitude of irreversible MTSES block is unchanged (Figure 6B). This is a demanded result if TEA and MTSES bind to the pore in an overlapping fashion. These findings do not reflect a decrease in the concentration of reactive MTSES, since TEA has no influence on the basal hydrolysis rate of MTSES in solution (Experimental Procedures). That TEA slows inhibition by MTSES but does not influence its final magnitude suggests that MTSES reaches its binding site only when TEA is unbound but can modify all its sites over time because TEA binding is reversible. This idea is supported by the observation that the kinetics of MTSES block are directly proportional to the fraction of TEA unblocked channels at equilibrium ( $f_u$ ). Thus,  $f_u$  is well described by the single-site isotherm:

$$(1) \quad cf_u = (1 + [\text{TEA}]/K_i)^{-1}$$

where  $[\text{TEA}]$  is the test concentration of TEA and  $K_i$  is the equilibrium inhibition constant for TEA block at the command voltage under study. Further, the MTSES blocking rate  $\tau^{-1}$  for A45C-minK is well described by:

$$(2) \quad (\tau)^{-1} = (\tau_0)^{-1} \cdot f_u$$

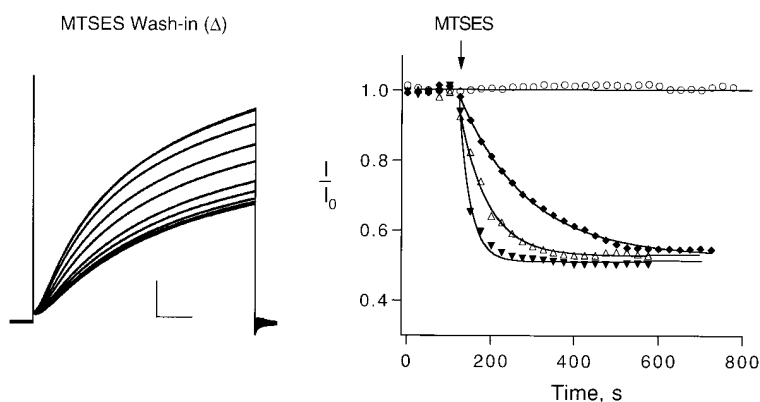


Figure 3. Kinetics of MTSES Block of A45C-MinK

Current traces (left) show successive pulses to 20 mV after wash-in of 3 mM MTSES by the protocol in Figure 2A; scale bars represent 0.2  $\mu$ A and 2 s. The plot (right) shows relaxation of currents measured after application of various concentrations of MTSES at 10 s into each test pulse ( $I$ , measured current;  $I_0$ , current before MTSES application). Protocol: after 6 control pulses, ND-96 bath solution was exchanged for MTSES solutions (MTSES) for 8 min (1 mM, diamonds), 5 min (3 mM, open triangles), or 3 min (10 mM, inverted triangles) and then changed back to ND-96, or perfused continuously with ND-96 (circles). Solid curves are single-exponential fits to the inhibition time-course; time constants are in Table 1.

where  $\tau$  and  $\tau_0$  are time constants in the presence and absence of TEA, respectively. Equation 2 indicates that a test concentration of TEA equal to its  $K_i$  should slow the rate of irreversible MTSES block twofold. In fact, the level of TEA predicted to halve the rate of MTSES block from the dose-response shown in Figure 6 is  $114 \pm 10$  mM, whereas the  $K_i$  for TEA block of A45C-minK at +20 mV calculated from tail current measurements is  $112 \pm 26$  mM (Table 1).

#### Binding Site Determinants for TEA and MTSES Overlap

Other support for the idea that this region coordinates TEA binding is that mutation to cysteine of positions 46, 47, 48, and 55 decreases TEA affinity  $40\% \pm 13\%$ ,  $82\% \pm 4\%$ ,  $24\% \pm 1\%$ , and  $120\% \pm 12\%$ , respectively

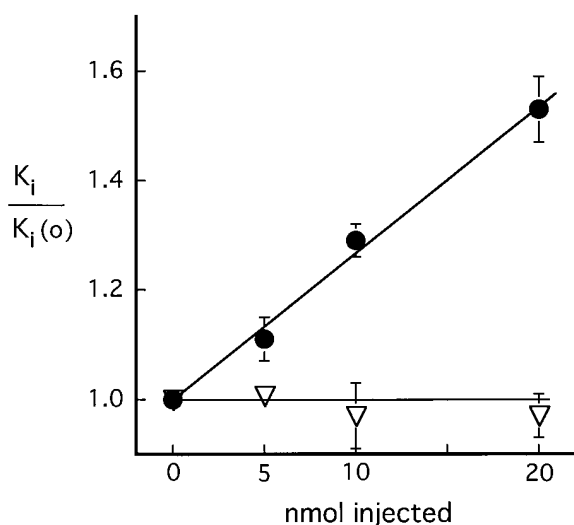


Figure 4. Effect of Injected KCl and NaCl on External TEA Blockade. Oocytes expressing wild-type minK were injected with 23 nl of aqueous salt solutions and whole-cell currents measured as in Figure 2A. The ratios of the equilibrium inhibition constants for external TEA blockade before ( $K_i(o)$ ) and after ( $K_i$ ) injection of 5, 10, or 20 nmol KCl (circles) or NaCl (inverted triangles) are plotted (mean  $\pm$  SD, 3–8 oocytes).

(3–5 oocytes). Further, reaction of MTSES with cysteine at positions 45 and 47, covalently modifying these side-chain moieties, decreases TEA affinity  $55\% \pm 10\%$  and  $53\% \pm 13\%$ , respectively (3–8 oocytes; see Figure 1). The gating of one mutant, Y47C-minK, is changed from wild type (see Figure 2A), and conclusions regarding the natural exposure of this site must be made with caution. Conversely, maintenance of wild-type  $K^+$ -selectivity, gating, and barium block in A45C-minK (Table 1) and the other 13 cysteine mutants (data not shown) supports the idea that the overall structure of these proteins is not grossly disrupted.

#### Discussion

##### Covalent Bond Formation Locates 3 MinK Residues in the Channel Pore

We show here that mutation to cysteine of minK positions 44, 45, and 47 renders channels susceptible to inhibition by MTSES via covalent modification. We show

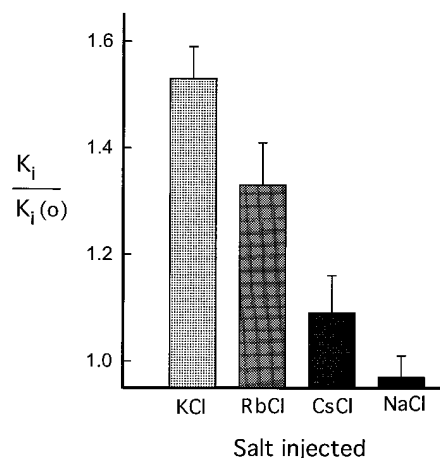


Figure 5. Differential Effects of Injected Monovalent Cation Salts on External TEA Affinity

The ratio of the equilibrium inhibition constants for external TEA blockade before ( $K_i(o)$ ) and after ( $K_i$ ) injection of 20 nmol of the indicated salt, measured as in Figure 4 (mean  $\pm$  SD, 4–8 oocytes).

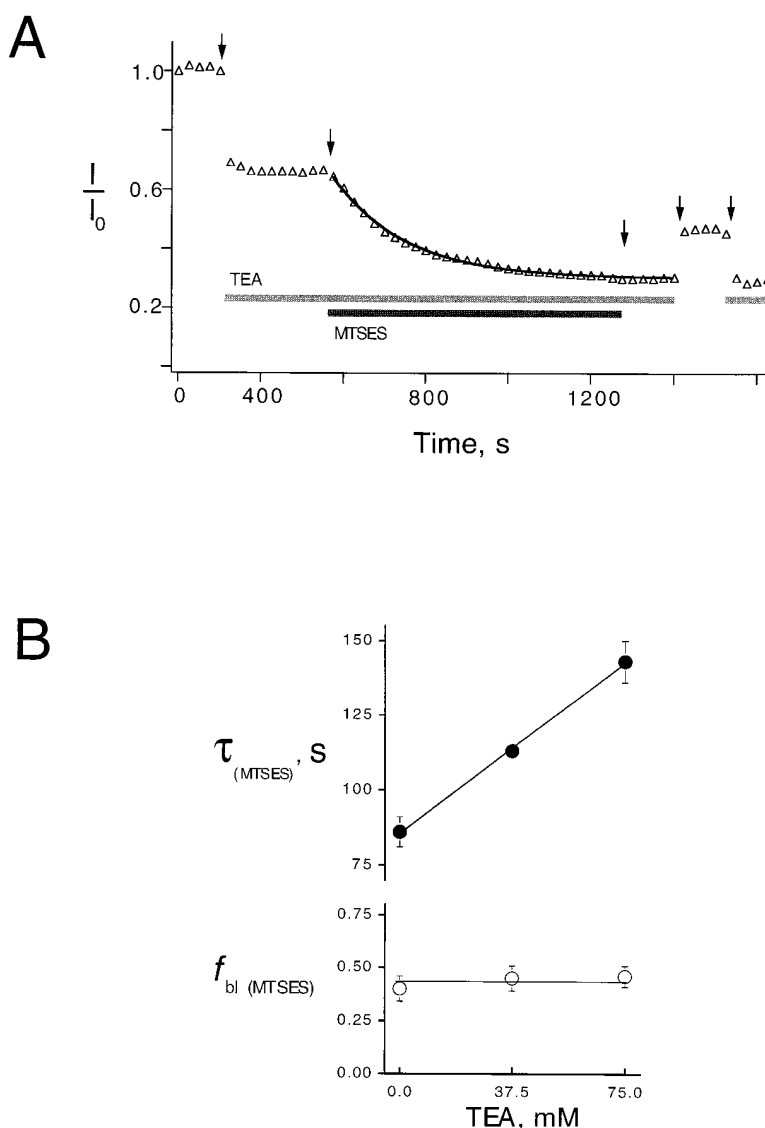


Figure 6. Effect of TEA on the Kinetics of MTSES Blockade of A45C-MinK

(A) Data for one oocyte; current was measured as in Figure 2A, first with constant flow of ND-96 and then during solution exchanges to 75 mM TEA solution (TEA bar), 3 mM MTSES and 75 mM TEA (MTSES bar), 75 mM TEA solution alone, ND-96, and 75 mM TEA solution.

(B) Effect of external TEA on the time constant for blockade by 3 mM MTSES ( $\tau$ , closed circles) and the fraction of irreversibly blocked current after MTSES washout ( $f_{bl}$ , open circles), mean  $\pm$  SD (4–12 oocytes). The time constant for MTSES block in the absence of TEA is  $86 \pm 5$  s (9 oocytes) and with 75 mM TEA is  $166 \pm 18$  s (10 oocytes).

that mutation of positions 46, 47, 48, and 55 alters reversible block by TEA, and we infer that TEA blocks minK by a direct pore-occlusion mechanism based on its sensitivity to transmembrane voltage and to permeant cations on the opposite side of the membrane. Finally, we show that TEA slows the rate but not the magnitude of MTSES blockade of A45C-minK channels and, reciprocally, that MTSES modification of A45C-minK decreases TEA affinity. These findings support the thesis that TEA and MTSES bind at interacting sites in an external ion channel vestibule and that minK residues contribute directly to these sites. The sites are “interacting” rather than “overlapping”, because TEA continues to block MTSES-modified channels, albeit with diminished affinity, and thus binding is not mutually exclusive.

Our results are not consistent with a purely regulatory role for minK (Attali et al., 1993). Moreover, the observation that native oocyte channels can be activated by over-expression of a wide variety of exogenous membrane proteins is now recognized to be the result of a

nonspecific process requiring levels of minK cRNA 1–2 orders of magnitude higher than used here (Shimbo et al., 1995; Tzounopoulos et al., 1995a).

#### Altering Intracellular Ionic Composition of Intact Oocytes by Microinjection

MinK is difficult to study in membrane patches because of its extremely small unitary conductance and low current density (Freeman and Kass, 1994; Yang and Sigworth, 1995). In addition, minK is sensitive to small changes in intracellular pH, calcium and other second messenger levels, and redox potential (Busch et al., 1992b, 1995; Yeh et al., 1994). Microinjection of salt solutions appears to address some of these experimental impediments by allowing reproducible whole-cell current measurements despite manipulation of intracellular ionic conditions. That it was possible to evaluate the effects of increasing internal KCl on external blockade of minK by TEA suggests that many aspects of the intracellular milieu remain stable despite the procedure.

Microinjection of KCl had predictable effects on  $K^+$  current magnitude and minK reversal potential. Based on reported estimates for oocyte solute space ( $\sim 400$  nL) and intracellular  $K^+$  concentration ( $\sim 90$  mM) (Lau et al., 1988), a 20 nmol KCl microinjection should raise intracellular  $K^+$  by  $\sim 50$  mM to 140 mM, leading to an 11 mV shift in  $E_K$  and a 55% increase in outward  $K^+$  current, close to the observed values. Microinjection to alter internal ionic composition may thus facilitate the study of other channels whose function is unstable in off-cell mode.

### MinK Pore Structure

We argue here that residues of the minK transmembrane domain contribute directly to formation of a  $K^+$ -selective ion conduction pathway based on identification of sites that mediate covalent pore blockade. This conclusion is harmonious with our prior studies indicating that positions 55 and 59 (also in the transmembrane domain) influence the permeability of monovalent cations relative to  $K^+$ , as well as blockade by  $Cs^+$  and TEA (Goldstein and Miller, 1991). Three inferences about minK structure can now be advanced. First, that positions 44, 45, and 47 are all accessible to MTSES argues that the N-terminal portion of the minK transmembrane segment does not have an  $\alpha$ -helical structure, as predicted (Takumi et al., 1988; Tzounopoulos et al., 1995a). Second, that MTSES blocks at positions 45 and 47 but not 46 argues that the later side chain is not in the aqueous phase. That TEA affinity is influenced by position 46 suggests that TEA may associate with the minK protein backbone at this site, a mechanism postulated to underlie coordination of  $K$  ions in Shaker-type  $K^+$  channels (Hidalgo and MacKinnon, 1995; Miller, 1995). Finally, that the negatively charged reagent MTSES can modify position 47 suggests that channel selection against anion conduction occurs at a more C-terminal minK location.

### A Working Model for MinK Channels

We have proposed that minK channels contain 2 minK monomers along with other, as-yet unidentified, non-minK subunits (Wang and Goldstein, 1995). Others have argued for a homomeric channel complex of 14 or more minK monomers (Tzounopoulos et al., 1995b). In support of a heteromeric channel model, Blumenthal and Kaczmarek (1994) argued that a non-minK subunit endogenous to oocytes and required for channel function (but present in only limited amounts) could explain the saturation of minK isochronal currents despite increasing levels of minK protein on the oocyte surface; we observed this behavior as well (Wang and Goldstein, 1995). The absence of minK-like currents in some cells expressing the protein is also consistent with a role for a non-minK subunit (Lesage et al., 1993). That MTSEA inhibits function of cysteine-free C107A-minK now provides direct support for the idea that another protein is critical to channel function and suggests it is a transmembrane protein exposed at the external membrane surface; its role in minK function is the subject of ongoing study.

### Experimental Procedures

#### Molecular Biology

MinK mutants were produced by polymerase chain reaction using mismatched oligonucleotides and pSD-minK, a modified pGEM-9ZF(−) vector (Promega) that carries a synthetic gene encoding rat minK (Hausdorff et al., 1991) between 5' and 3' untranslated segments that favor high levels of channel expression in oocytes (Wang and Goldstein, 1995). Mutants were confirmed by DNA sequencing, and cRNA was synthesized as previously described (Wang and Goldstein, 1995). All cysteine point mutants were otherwise cysteine free (C107A-minK).

#### Electrophysiology

Oocytes were isolated from *Xenopus laevis* (Nasco, Atkinson, WI), defolliculated by collagenase treatment, and injected the next day with 46 nL containing 2 ng cRNA (Wang and Goldstein, 1995). Whole-cell currents were measured 2 or 3 days after cRNA injection with constant perfusion by two-electrode voltage clamp. Experiments were performed using a GeneClamp 500 (Axon Instruments), an ITC-16 A/D converter and Pulse software (Instrutech, NY), and a Macintosh Quadra 800 computer. Data were filtered at 250 Hz and sampled at 1 kHz. Perfusion was controlled manually with an exchange time  $< 5$  s. To study the effects of changing intracellular salt composition, oocytes were microinjected with unbuffered aqueous solutions of KCl, RbCl, CsCl, and NaCl and studied 30 to 120 min thereafter. ND-96 solution contains 96 mM NaCl, 2 mM KCl, 1 mM  $MgCl_2$ , 0.3 mM  $CaCl_2$ , and 5 mM HEPES (pH 7.6). TEA chloride was isotonicly substituted for NaCl in ND-96.

#### Methylthiosulfonate-ethylsulfonate (MTSES) and ethylammonium (MTSEA)

MTSES and MTSEA solutions were made freshly from powdered sodium or bromide salt, respectively (Toronto Research Chemicals, Ontario, Canada), by isotonic substitution for NaCl in ND-96 and used within 0 to 15 min of dissolving. The rate of MTSES hydrolysis in aqueous solution was found to be the same in the absence and presence of TEA by the method of Stauffer and Karlin (1994); hydrolysis half-times in ND-96 and 75 mM TEA solution were  $57 \pm 11$  min and  $61 \pm 11$  min, respectively (mean  $\pm$  SD,  $n = 5$ ). When 2.5 mM MTSEA was applied to C107A-minK for 3 min by the protocol of Figure 3,  $52\% \pm 6\%$  (mean  $\pm$  SEM) of the current was blocked after MTSEA washout (5 oocytes).

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